Litton BIONETICS

Summary of mutagenicity screening studies, host-mediated assay cytogenetics dominant lethal assay-Contract FDA 71-268 & Compound FDA 71-35 (**Sodium Thiosulfate**)
6/28/73 (1st Revised: 10/9/73) (2nd Revised: 11/20/73)

SCREENING STUDIES
Contract FDA 71-268
FDA 71-35
SODIUM THIOSULFATE
HOST-MEDIATED ASSAY
CYTOGENETICS
DOMINANT LETHAL ASSAY

M 30

5515 Wisconsin Avenue
Bethesda, Maryland
20014

M 30

SUMMARY OF MUTAGENICITY SCREENING STUDIES

Contract FDA 71-268
FDA 71-35
SODIUM THIOSULFATE
HOST-MEDIATED ASSAY
CYTOGENETICS
DOMINANT LETHAL ASSAY

SUBMITTED TO

Food & Drug Administration
Department of Health, Education and Welfare
Rockville, Maryland

SUBMITTED BY

Litton Bionetics, Inc.
7315 Wisconsin Avenue
Bethesda, Maryland

June 28, 1973
(October 9, 1973 - Revised)

(November 20, 1973 - Revised)

Litton BIONETICS

Litton BIONETICS

7315 Wisconsin Avenue, Bethesda, Maryland 20014 301 652-6616

April 2, 1974

Mr. William L. Totten
Contracting Officer
Negotiated Contracts Branch
Division of Contracts & Grants Management
Food & Drug Administration, HFA-510
5600 Fishers Lane, Room 4C-25
Rockville, Maryland 20852

Reference: Contract FDA 71-268; LBI's Project #2446

Dear Mr. Totten:

Litton Bionetics, Inc. is pleased to submit a report for the referenced contract entitled "Mutagenicity Screening Studies" for compound FDA 71-35, Sodium Thiosulfate.

Included in this report are the results and raw data of the three tests conducted: The Host-Mediated Assay; the Cytogenetic Studies; and the Dominant Lethal Assay. Eight (8) copies are being submitted for your review.

If there are any questions concerning this report, or, if additional information is required, please do not hesitate to contact me.

Sincerely,

LITTON BIONETICS, INC.

Robert J. Weir

Robert J. Weir, Ph.D.
Vice President

RJW:lls
Enclosures (8)

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I. REPORT

A. Introduction

Litton Bionetics, Inc. has investigated the possible mutagenicity of compounds selected and provided by the Food & Drug Administration under Contract 71-268. LBI's investigation utilized the three mammalian test systems herein described--Host-Mediated Assay, Cytogenetic studies and Dominant Lethal Assay. These tests provide information as to the types of genetic damage caused by environmental compounds -- pesticides, chemicals, food additives, drugs, and cosmetics.

The Host-Mediated Assay is based upon the assumption that the action of a mutagen on the genetics of bacteria is similar to that in man. This is further strengthened by the use of an eukaryotic organism (e. g., Saccharomyces cerevisiae). Since the mutation frequencies are well established for the indicator organism, any deviation due to the action of the test compound is readily detectable. As some compounds are mutagenic in bacteria and not in the host animal, and vice versa, this test is able to differentiate an action which may have been due to hosts' ability to detoxify or potentiate a suspected mutagen. This action is dependent upon the ability of the compound to gain access to the peritoneal cavity. Coupled with the direct action of the compound on the indicator organism in vitro, the assay provides a clear insight into host mediation of mutagenicity.

Cytogenetics provides a valuable tool for the direct observation of chromosomal damage in somatic cells. Alteration of the chromosome number and/or form in somatic cells may be an index of mutation. These studies utilized examination of bone marrow cells arrested in C-metaphase from rats treated with test compound as compared to positive and negative control animals. If muta-

tional changes occur the types of damage expected due to the action of chemicals are structural rearrangements, breaks and other forms of damage to the chromosomal complement of the cells exposed.

For the in vitro cytogenetic studies, we have a more rapid and inexpensive means of determining chromosomal damage. This is accomplished by observing cells in anaphase. As the chromatids separate and move along the spindle, aberrations may occur. Chromatids which do not migrate to the daughter cells may lead to uneven distribution of parts or of entire chromatids (mitotic nondysjunction). These give rise to "side arm" bridges which have been interpreted as point stickiness or localized failures of chromosome duplication point errors. These aberrations (bridges, pseudochiasmata, multipolar cells, acentric fragments, etc.) are extremely sensitive indicators of genetic damage.

The Dominant Lethal Test is an accurate and sensitive measure of the amount and type of fetal wastage which may occur following administration of a potential mutagen. Dominant lethal mutations are indicators of lethal genetic lesions. The effects of mutagens on the chromosomal complement of the spermatozoa of treated males results in alterations of form and number of chromosomes. Structural rearrangements and aneuploidy may lead to the production of non-viable zygotes, early and late fetal deaths, abortions

and congenital malformations. In addition, aberrations could lead to sterility or reduced reproductive capacity of the F_1 generation. The action of a mutagen on specific portions of spermatogenesis is also apparent in this test.

B. Objective

The purpose of these studies is to determine any mutagenic effect of the test compound by employing the Host Mediated Assay, Cytogenetics Studies and the Dominant Lethal Assay, both in vivo and in vitro tests are employed with the cytogenetic and microbial test systems. These tests and their descriptions are referenced in the Appendices A through F.

C. Compound

1. Test Material

Compound FDA 71-35, Sodium Thiosulfate as supplied by the Food and Drug Administration.

2. Dosages

The animals employed, the determination of the dosage levels and the route of administration are contained in the technical discussions.

The dosage levels employed for compound FDA 71-35 are as follows for Cytogenetics Studies in vivo in rats.

Low Level	50 mg/kg
Intermediate Level	500 mg/kg
LD_5	5000 mg/kg
Negative Control	saline
Positive Control (TEM*)	0.3 mg/kg

The dosage levels employed for compound FDA 71-35 are as follows for Host Mediated Assay in vivo in mice.

Low Level	50 mg/kg
Intermediate Level	500 mg/kg
LD_5	5000 mg/kg
Negative Control	saline
Positive Control	
(EMS **)	350 mg/kg
(DMN***)	100 mg/kg

* Triethylene Melamine
** Ethyl Methane Sulfonate
*** Dimethyl Nitrosamine

The dosage levels employed for compound FDA 71-35 are as follows for the Dominant Lethal Assay in vivo in rats.

Low Level	50	mg/kg
Intermediate Level	500	mg/kg
LD_5	5000	mg/kg
Negative Control	saline	
Positive Control (TEM*)	0.3	mg/kg

The in vitro cytogenetics studies were performed employing three logarithmic dose levels.

Low Level	8	mcg/ml
Medium Level	80	mcg/ml
High Level	800	mcg/ml
Negative Control	saline	
Positive Control (TEM*)	0.1	mcg/ml

The discussion of this test is contained in the technical discussions.

D. Methods

The protocols employed are explained in Appendices C and D.

E. Summary

1. Host Mediated Assay

This compound caused no significant increases in mutant frequencies with Salmonella TA-1530 or G-46. Studies with Saccharomyces D-3 were negative except the acute high which was doubled in recombinant frequency. In vitro tests were negative.

2. Cytogenetics

(a) In vivo - The compound produced no detectable significant aberration of the bone marrow metaphase chromosomes of rats when administered orally at the dosage levels employed in this study.

(b) In vitro - The compound produced no significant aberration in the anaphase chromosomes of human tissue culture cells when tested at the dosage levels employed in this study.

3. Dominant Lethal

This compound was considered to be non-mutagenic in this assay system when used at the dosage levels employed in this study in rats.

F. Results and Discussion

1. Toxicity

a. In vivo

Ten (10) male albino rats with an average body weight of 285 grams were given compound FDA 71-35. The compound was dissolved in a solution of 0.85% saline and was administered by gastric intubation at a dosage of 5,000 mg/kg of body weight. The animals received two (2) intubations (2 ml per intubation totaling 4 ml per rat) of the compound on the first day of study. All animals appeared normal during treatment and for an additional six (6) days post-treatment observation. Necropsies of these animals were performed on day seven (7) and revealed no gross morphological changes in the organs examined.

b. In vitro

The compound was dissolved in a solution of 0.85% saline at the concentration listed. It was introduced into culture tubes containing WI-38 cells in a logarithmic phase of growth. The cells were observed for cytopathic effect (CPE) and the presence of mitoses at 24 and 48 hours.

Tube No.	No. of cells	Conc. mcg/ml	CPE	Mitosis
1		1000	+	+
2		1000	+	±
3		500	-	+
4		500	-	+
5		100	-	+
6		100	-	+
7		50	-	+
8		50	-	+
9		10	-	+
10		10	-	+

Since a CPE was observed at 1000 mcg/ml a closer range of concentrations was employed as follows.

1		1000	+	+
2		1000	±	+
3		800	-	+
4		800	-	+
5		600	-	+
6		600	-	+
7		400	-	+
8		400	-	+
9		200	-	+
10		200	-	+

The high level employed was 800 mcg/ml, the intermediate level was 80 mcg/ml and the low level was 8.0 mcg/ml.

c. Toxicity data

This compound was administered at an extremely high concentration of 5,000 mg/kg with no abnormal effect observed in the animals.

Solvent: 0.85% saline

Dosage Form: Solution

Animals: Male rats with an average body weight of 285 grams.

All animals were observed for seven (7) days.

Range Findings:

Dose	No. Dead/No. Animals	Necropsy and Day of Death
5,000 mg/kg	0/10	None

There were no abnormal gross pathology findings in the animals dosed at 5,000 mg/kg, and a determination of an LD_{50} was not performed. The LD_{50} is assumed to be greater than 5,000 mg/kg.

2. Host-Mediated Assay

Compound FDA 71-35 showed no significant increases in mutant frequencies with *Salmonella* TA-1530 and G-46. All dose levels with *Saccharomyces* D-3 were negative except the acute high dose which showed a doubling of recombinant frequency which may or may not be significant. Additional testing will be needed to determine whether or not the compound is actually recombinogenic. *In vitro* tests were negative.

EVALUATION SHEET

Compound: 71-35 Sodium Thiosulfate

Indicator Strain	In Vitro	In Vivo: Possible Low Recoveries	In Vivo: Controls	Other Comments
TA-1530	pos.	NC PC AL AI (AH) SANC SAL SAI SAH	NC OK PC OK SANC OK	1. No doses positive 2. Acute high possibly low recovery but the reversion frequency not significant from the control: Elevated slightly.
NC Acutes { 11/3/72	(neg.)			
PC 11/1/72				
SANC Subacutes { 7/21/72				
SMPC 7/11/72				
G-46	pos.	NC PC AL AI AH SANC SAL (SAI) SAH	NC OK PC OK SANC OK	1. Positive control might be a little low - but I think it is acceptable. 2. Subacute intermediate dose has low recovery but again there is no significant change in the reversion frequency resulting from the low recovery (slight elevation in freq.) 3. No doses positive
NC Acutes { 7/31/72	(neg.)			
SANC Subacutes { 8/4/72				
D3	pos.	NC PC AL AI AH SANC SAL SAI SAH	NC OK PC OK SANC OK	1. Acute high dose shows a solid doubling and may be positive.
NC PC Acutes { 6/26/72	(neg.)			
SMNC Subacutes { 6/30/72				

Summary: The data in this report should be acceptable. The fact that two doses are slightly low in recoveries should not be significant as the results for those doses are in line with all other doses for that experiment. The doubling exhibited in the D3 acute high dose may or may not be significant. Additional tests would be necessary to determine if the compound is recombinogenic.

David Brusick

a. HOST MEDIATED ASSAY SUMMARY SHEETS

FDA CONTRACT 71-268

FDA COMPOUND 71-35

SODIUM THIOSULFATE

HOST MEDIATED ASSAY

SUMMARY SHEET

OUTLIERS INCLUDED

COMPOUND: FDA 71-35

	SALMONELLA TA1530		SALMONELLA G-46		SACCHAROMYCES D-3	
	MMF (X 10E-8)	MFT/MFC	MMF (X 10E-8)	MFT/MFC	MRF (X 10E-5)	MRT/MRC
ACUTE						
NC	.58		.84		5.22	
PC	7.60	13.10	16.19	19.27	42.53	8.15
AL	.87	1.50	1.89	2.25	5.51	1.06
AI	.50	.86	.76	.90	5.31	1.02
AH	1.59	2.74	1.39	1.65	11.66	2.23
SUBACUTE						
NC	.89		.69		4.69	
SL	1.93	2.17	1.71	2.48	3.62	.77
SI	.95	1.07	1.10	1.59	4.21	.90
SH	1.36	1.53	.79	1.14	3.85	.82

IN VITRO	TA1530	G-46	D-3 % CONC	D-3 % SURVIVAL	D-3 R X 10E5
TCPD	-	-	0.25	71.3	16
NC	-	-	-	100.0	5
PC	+	+	0.50	68.8	267

STOP
SRU'S:.6
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HOST MEDIATED ASSAY

SUMMARY SHEET

OUTLIERS REMOVED

COMPOUND: FDA 71-35

	SALMONELLA TA1530		SALMONELLA G-46		SACCHAROMYCES D-3	
	MMF (X 10E-8)	MFT/MFC	MMF (X 10E-8)	MFT/MFC	MRF (X 10E-5)	MRT/MRC
ACUTE						
NC	.56		.67		5.22	
PC	7.60	13.10	13.29	19.84	42.53	8.15
AL	.66	1.17	1.33	1.98	5.51	1.06
AI	.50	.86	.76	1.13	5.31	1.02
AH	1.30	2.24	1.39	2.07	10.25	1.96
SUBACUTE						
NC	.75		.69		5.07	
SL	1.93	2.57	1.38	2.00	3.62	.71
SI	.95	1.27	1.10	1.59	4.21	.83
SH	1.36	1.81	.79	1.14	3.85	.76

IN VITRO	TA1530	G-46	D-3 % CONC	D-3 % SURVIVAL	R X 10E5
NC					
PC					

b. HOST MEDIATED ASSAY DATA SHEETS

FDA CONTRACT 71-268

FDA COMPOUND 71-35

SODIUM THIOSULFATE

Host Mediated Assay - Adjusted Raw CFU x 10^7/0.6 ml

The true raw colony counts were lost through automation for this compound. Thus, the source of the adjusted raw CFU x 10^7/0.6 ml (Column A) was the true raw counts as assimilated by the automatic colony counter, multiplied by the automatic program by 0.16666666666667 (Column B) and then divided by 0.1667 (the check figure). The original concept was that the true CFU x 10^7/0.6 ml would be printed as column A. Through a programing anomaly the Column B check figure was obtained as the raw CFU x 10^7/0.6 ml and recorded as such.

Step 1: Technician set counter - plates on counter.
Step 2: Automatic equipment accumulates counts on 3 plates of 10^{-6} dilution as CFU x 10^7/0.6 ml.
Step 3: Automatic equipment multiplies count obtained in step 1 by 0.16666666666667 to obtain total count/ml at 10^8.
Step 4: Automatic check of result of step 3.
TC x 10^8 ÷ 0.1667 = CFU x 10^7/0.6 ml
Step 5: Technician was to record the true raw CFU x 10^7/0.6 ml in log book, however, through error the computer provided the Column B check figure as the raw count.

To clarify the problem Column A is headed Adjusted Raw CFU X $10E^7$/ 0.6 ml in each case where the check figure was provided as the raw count.

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-55

ORGANISM: SALMONELLA TA1530

DOSE LEVEL: NEGATIVE CONTROL - SALINE

TREATMENT: IN VIVO, ORAL, ACUTE

DATE STARTED: NOVEMBER 3, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	31.70	5.28	3.00	.57
2	35.20	5.87	2.00	.34
3	21.70	3.62	2.00	.55
4	47.00	7.83	1.00	.13
5	27.10	4.52	4.00	.89
6	17.60	2.93	3.00	1.02
7	22.50	3.75	2.00	.53
8	34.00	5.67	3.00	.53
9	43.80	7.30	5.00	.68

NO. OF ANIMALS EQUALS 9
SAMPLES WITH ZERO MUTANTS EQUAL 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	5.20	2.78	.58
RANGE	4.90	4.00	.90
MAX	7.83	5.00	1.02
MIN	2.93	1.00	.13

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA TA153

DOSE LEVEL: POSITIVE CONTROL - DMN - 100 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: NOVEMBER -, 19?2

ANIMAL NUMBER	A RAW CFU X 10E7/0.2ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	42.70	7.12	52.00	7.31
2	35.00	5.83	41.00	7.03
3	29.70	4.95	38.00	7.68
4	46.30	7.72	61.00	7.90
5	22.00	3.67	33.00	9.00
6	24.50	4.08	40.00	9.80
7	37.70	6.28	29.00	4.62
8	28.00	4.67	22.00	4.71
9	26.00	4.33	31.00	7.15
10	34.90	5.82	63.00	10.83

NO. OF ANIMALS EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	5.45	41.00	7.60
RANGE	4.05	41.00	6.22
MAX	7.72	63.00	10.83
MIN	3.67	22.00	4.62

NO OUTLIERS

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35

ORGANISM: SALMONELLA TA1530

DOSE LEVEL: LOW - 50 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE

DATE STARTED: NOVEMBER 3, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	13.50	2.25	1.00	.44	
2	30.00	5.00	4.00	.80	
3	16.70	2.78	2.00	.72	
4	47.80	7.97	4.00	.50	
5	8.00	1.33	3.00	2.25	*
6	50.10	8.35	5.00	.60	
7	33.10	5.52	6.00	1.09	
8	40.70	6.78	4.00	.59	

NO. OF ANIMALS EQUALS 8
TOTAL CFU OUT OF RANGE EQUALS 2

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	5.00	3.63	.87
RANGE	7.02	5.00	1.81
MAX	8.35	6.00	2.25
MIN	1.33	1.00	.44

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	5.52	3.71	.68
RANGE	6.10	5.00	.64
MAX	8.35	6.00	1.09
MIN	2.25	1.00	.44

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35

ORGANISM: SALMONELLA TA1530

DOSE LEVEL: INTERMEDIATE - 500 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE

DATE STARTED: NOVEMBER 3, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	59.40	9.90	2.00	.20
2	7.30	1.22	1.00	.82
3	25.10	4.18	1.00	.24
4	26.00	4.33	1.00	.23
5	42.50	7.08	5.00	.71
6	32.20	5.37	4.00	.75
7	32.00	5.33	3.00	.56

NO. OF ANIMALS EQUALS 7
TOTAL CFU OUT OF RANGE EQUALS 2
SAMPLES WITH ZERO MUTANTS EQUAL 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	5.35	2.43	.50
RANGE	8.68	4.00	.62
MAX	9.90	5.00	.82
MIN	1.22	1.00	.20

NO OUTLIERS

[illegible] MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-55 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: NOVEMBER 3, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	10.20	1.70	2.00	1.18	
2	5.40	1.07	1.00	.94	
3	22.70	3.78	6.00	1.59	
4	8.20	1.37	5.00	3.66	*
5	10.00	1.67	1.00	.60	
6	15.30	2.55	4.00	1.57	
7	10.00	1.67	3.00	1.80	
8	12.70	2.12	3.00	1.42	

NO. OF ANIMALS EQUALS 8
TOTAL CFU OUT OF RANGE EQUALS 2

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	1.99	3.13	1.59
RANGE	2.72	5.00	3.06
MAX	3.78	6.00	3.66
MIN	1.07	1.00	.60

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.08	2.86	1.30
RANGE	2.72	5.00	1.20
MAX	3.78	6.00	1.80
MIN	1.07	1.00	.60

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HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: NEGATIVE CONTROL - SALINE (SUBACUTE TRIALS)

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JULY 21, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	20.10	3.35	2.00	.60	
2	12.24	2.04	1.00	.49	
3	21.18	3.53	3.00	.85	
4	9.54	1.59	1.00	.63	
5	12.60	2.10	1.00	.48	
6	12.12	2.02	3.00	1.49	
7	10.98	1.33	1.00	.55	
8	14.88	2.48	2.00	.81	
9	14.10	2.35	5.00	2.13	*
10	27.18	4.53	4.00	.88	

NO. OF ANIMALS EQUALS 10

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.58	2.30	.89
RANGE	2.94	4.00	1.65
MAX	4.53	5.00	2.13
MIN	1.59	1.00	.48

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.61	2.00	.75
RANGE	2.94	3.00	1.01
MAX	4.53	4.00	1.49
MIN	1.59	1.00	.48

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: POSITIVE CONTROL - DMN - 100 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JULY 17, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	21.42	3.57	18.00	5.04	
2	9.30	1.55	11.00	7.10	
3	51.42	8.57	28.00	3.27	
4	10.20	1.70	22.00	12.94	*
5	17.52	2.92	15.00	5.14	
6	25.02	4.17	36.00	8.63	
7	25.68	4.28	42.00	9.81	
8	31.50	5.25	37.00	7.05	
9	15.78	2.63	17.00	6.46	

NO. OF ANIMALS EQUALS 9
TOTAL CFU OUT OF RANGE EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.85	25.11	7.27
RANGE	7.02	31.00	9.67
MAX	8.57	42.00	12.94
MIN	1.55	11.00	3.27

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	4.12	25.50	6.56
RANGE	7.02	31.00	6.55
MAX	8.57	42.00	9.81
MIN	1.55	11.00	3.27

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: LOW - 50 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: JULY 21, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	13.14	2.19	5.00	2.28
2	21.90	3.65	6.00	1.64
3	21.78	3.63	4.00	1.10
4	13.08	2.18	6.00	2.75
5	14.70	2.45	6.00	2.45
6	18.18	3.03	2.00	.66
7	13.50	2.25	6.00	2.67
8	13.02	2.17	3.00	1.38
9	17.46	2.91	7.00	2.41

NO. OF ANIMALS EQUALS 9
TOTAL CFU OUT OF RANGE EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.72	5.00	1.93
RANGE	1.48	5.00	2.09
MAX	3.65	7.00	2.75
MIN	2.17	2.00	.66

NO OUTLIERS

...OST MEDIATED ASSAY REPORT SHEET

...POUND: FDA 71-35 ORGANISM: SALMONELLA TA1530

...OSE LEVEL: INTERMEDIATE - 500 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: JULY 21, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	20.34	3.39	4.00	1.18
2	11.94	1.99	1.00	.50
3	9.18	1.53	2.00	1.31
4	23.82	3.97	5.00	1.26
5	21.90	3.65	3.00	.82
6	20.10	3.35	3.00	.90
7	22.50	3.75	4.00	1.07
8	9.48	1.58	2.00	1.27
9	23.88	3.98	3.00	.75
10	14.10	2.35	1.00	.43

NO. OF ANIMALS EQUALS 10

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.95	2.80	.95
RANGE	2.45	4.00	.88
MAX	3.98	5.00	1.31
MIN	1.53	1.00	.43

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: JULY 21, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	19.38	3.23	8.00	2.48
2	21.72	3.62	5.00	1.38
3	22.14	3.69	5.00	1.35
4	12.54	2.09	7.00	3.35
5	22.14	3.69	2.00	.54
6	13.62	2.27	1.00	.44
7	14.10	2.35	1.00	.43
8	20.34	3.39	3.00	.88

NO. OF ANIMALS EQUALS 8
NO. OF DEAD ANIMALS EQUALS 1
NO. OF CONTAMINATED EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.04	4.00	1.36
RANGE	1.60	7.00	2.92
MAX	3.69	8.00	3.35
MIN	2.09	1.00	.43

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA G-46

DOSE LEVEL: NEGATIVE CONTROL - SALINE

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JULY 31, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	11.10	1.85	1.00	.54	
2	20.82	3.47	2.00	.58	
3	8.94	1.49	3.00	2.01	*
4	14.70	2.45	1.00	.41	
5	18.30	3.05	3.00	.98	
6	14.34	2.39	2.00	.84	
7	15.54	2.59	2.00	.77	
8	9.96	1.66	1.00	.60	

NO. OF ANIMALS EQUALS 8
NO. OF CONTAMINATED EQUALS 1
SAMPLES WITH ZERO MUTANTS EQUAL 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.37	1.88	.84
RANGE	1.98	2.00	1.61
MAX	3.47	3.00	2.01
MIN	1.49	1.00	.41

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.49	1.71	.67
RANGE	1.81	2.00	.58
MAX	3.47	3.00	.98
MIN	1.66	1.00	.41

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA G-46

DOSE LEVEL: POSITIVE CONTROL - DMN - 100 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JULY 31, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	6.24	1.04	44.00	42.31	*
2	22.74	3.79	56.00	14.78	
3	8.94	1.49	38.00	25.50	
4	18.18	3.03	37.00	12.21	
5	19.32	3.22	35.00	10.87	
6	14.53	2.42	30.00	12.39	
7	25.75	4.29	48.00	11.18	
8	13.62	2.27	22.00	9.69	
9	33.70	5.62	67.00	11.93	
10	18.50	3.08	34.00	11.03	

NO. OF ANIMALS EQUALS 10

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.03	41.10	16.19
RANGE	4.58	45.00	32.62
MAX	5.62	67.00	42.31
MIN	1.04	22.00	9.69

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.25	40.78	13.29
RANGE	4.13	45.00	15.81
MAX	5.62	67.00	25.50
MIN	1.49	22.00	9.69

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FJA 71-35 ORGANISM: SALMONELLA G-46

DOSE LEVEL: LOW - 50 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JULY 31, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	30.42	5.07	8.00	1.58	
2	18.52	3.09	2.00	.65	
3	24.30	4.05	3.00	.74	
4	16.50	2.75	2.00	.73	
5	9.48	1.58	3.00	1.90	
6	12.30	2.05	13.00	6.34	*
7	14.94	2.49	10.00	4.02	
8	15.54	2.59	2.00	.77	
9	23.70	3.95	1.00	.25	

NO. OF ANIMALS EQUALS 9
TOTAL CFU OUT OF RANGE EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.07	4.89	1.89
RANGE	3.49	12.00	6.09
MAX	5.07	13.00	6.34
MIN	1.58	1.00	.25

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.20	3.88	1.33
RANGE	3.49	9.00	3.76
MAX	5.07	10.00	4.02
MIN	1.58	1.00	.25

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA G-46

DOSE LEVEL: INTERMEDIATE - 500 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JULY 31, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	15.54	2.59	1.00	.39
2	10.70	1.78	2.00	1.12
3	12.78	2.13	1.00	.47
4	12.90	2.15	1.00	.47
5	24.54	4.09	2.00	.49
6	14.10	2.35	3.00	1.28
7	12.30	2.05	2.00	.98
8	12.42	2.07	2.00	.97
9	10.98	1.83	1.00	.55
10	20.34	3.39	3.00	.88

NO. OF ANIMALS EQUALS 10

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.44	1.80	.76
RANGE	2.31	2.00	.89
MAX	4.09	3.00	1.28
MIN	1.78	1.00	.39

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-55

ORGANISM: SALMONELLA G-46

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE

DATE STARTED: JULY 31, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	13.74	2.29	2.00	.87
2	11.94	1.99	4.00	2.01
3	7.20	1.20	3.00	2.50
4	11.82	1.97	2.00	1.02
5	16.02	2.67	2.00	.75
6	30.90	5.15	3.00	.58
7	7.50	1.25	3.00	2.40
8	16.74	2.79	2.00	.72
9	14.94	2.49	2.00	.80
10	13.38	2.23	5.00	2.24

NO. OF ANIMALS EQUALS 10

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.40	2.80	1.39
RANGE	3.95	3.00	1.92
MAX	5.15	5.00	2.50
MIN	1.20	2.00	.58

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA G-46

DOSE LEVEL: NEGATIVE CONTROL - SALINE (SUBACUTE TRIALS)

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: AUGUST 4, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	19.80	3.30	2.00	.61
2	21.12	3.52	2.00	.57
3	6.90	1.15	1.00	.87
4	17.10	2.85	3.00	1.05
5	8.58	1.43	1.00	.70
6	19.86	3.31	2.00	.60
7	8.94	1.49	1.00	.67
8	13.38	2.23	1.00	.45

NO. OF ANIMALS EQUALS 8
SAMPLES WITH ZERO MUTANTS EQUAL 2

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.41	1.63	.69
RANGE	2.37	2.00	.60
MAX	3.52	3.00	1.05
MIN	1.15	1.00	.45

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA G-46

DOSE LEVEL: LOW - 50 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: AUGUST 4, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	7.20	1.20	3.00	2.50	
2	13.32	2.22	1.00	.45	
3	11.70	1.95	4.00	2.05	
4	16.50	2.75	1.00	.36	
5	10.74	1.79	4.00	2.23	
6	11.70	1.95	5.00	2.56	
7	9.72	1.62	7.00	4.32	*
8	24.72	4.12	2.00	.49	
9	30.30	5.05	2.00	.40	

NO. OF ANIMALS EQUALS 9
TOTAL CFU OUT OF RANGE EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.52	3.22	1.71
RANGE	3.85	6.00	3.96
MAX	5.05	7.00	4.32
MIN	1.20	1.00	.36

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.63	2.75	1.38
RANGE	3.85	4.00	2.20
MAX	5.05	5.00	2.56
MIN	1.20	1.00	.36

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA G-46

DOSE LEVEL: INTERMEDIATE - 500 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: AUGUST 4, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	6.30	1.05	2.00	1.90
2	25.32	4.22	2.00	.47
3	8.70	1.45	1.00	.69
4	12.00	2.00	1.00	.50
5	11.70	1.95	1.00	.51
6	5.10	1.35	3.00	2.22
7	6.72	1.12	2.00	1.79
8	6.54	1.09	1.00	.92
9	13.38	2.23	2.00	.90

NO. OF ANIMALS EQUALS 9
TOTAL CFU OUT OF RANGE EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	1.83	1.67	1.10
RANGE	3.17	2.00	1.75
MAX	4.22	3.00	2.22
MIN	1.05	1.00	.47

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SALMONELLA G-46

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: AUGUST 4, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	55.14	9.19	7.00	.76
2	42.12	7.02	3.00	.43
3	20.94	3.49	4.00	1.15
4	19.14	3.19	3.00	.94
5	58.68	9.78	5.00	.51
6	7.32	1.22	1.00	.82
7	7.50	1.25	1.00	.80
8	24.96	4.16	3.00	.72
9	6.54	1.09	1.00	.92
10	49.14	8.19	7.00	.85

NO. OF ANIMALS EQUALS 10

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	4.86	3.50	.79
RANGE	8.69	6.00	.72
MAX	9.78	7.00	1.15
MIN	1.09	1.00	.43

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: NEGATIVE CONTROL - SALINE

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JUNE 26, 1972

ANIMAL NUMBER	A RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5
1	319.00	.32	.00	.00
2	410.00	.41	1.00	2.44
3	213.00	.21	1.00	4.69
4	452.00	.45	2.00	4.42
5	223.00	.22	2.00	8.97
6	329.00	.33	3.00	9.12
7	376.00	.38	2.00	5.32
8	414.00	.41	3.00	7.25
9	350.00	.35	1.00	2.86
10	362.00	.36	3.00	8.29
TOTAL		3.45	18.00	

NO. OF ANIMALS EQUALS 10

MEAN C/MEAN B = 5.22

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.34	1.80	5.34
RANGE	.24	3.00	9.12
MAX	.45	3.00	9.12
MIN	.21	.00	.00

NO OUTLIERS

...OST MEDIATED ASSAY REPORT SHEET

...POUND: FDA 71-55 ORGANISM: SACCHAROMYCES D-3

...OSE LEVEL: POSITIVE CONTROL - EMS - 350 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JUNE 26, 1972

ANIMAL NUMBER	A RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5
1	328.00	.33	13.00	39.63
2	285.00	.28	15.00	52.63
3	820.00	.82	48.00	58.54
4	351.00	.35	15.00	42.74
5	309.00	.31	12.00	38.83
6	443.00	.44	12.00	27.09
7	404.00	.40	16.00	39.60
8	327.00	.33	14.00	42.81
9	940.00	.94	33.00	35.11
10	331.00	.33	15.00	45.32
TOTAL		4.54	193.00	

...O. OF ANIMALS EQUALS 10

MEAN C/MEAN B = 42.53

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.45	19.30	42.23
RANGE	.65	35.00	31.45
MAX	.94	48.00	58.54
MIN	.28	13.00	27.09

...O OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: LOW - 50 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JUNE 26, 1972

ANIMAL NUMBER	A RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5
1	273.00	.27	3.00	10.99
2	611.00	.61	2.00	3.27
3	472.00	.47	3.00	6.36
4	421.00	.42	3.00	7.13
5	560.00	.56	5.00	8.93
6	861.00	.86	4.00	4.65
7	492.00	.49	2.00	4.07
8	621.00	.62	3.00	4.83
9	407.00	.41	1.00	2.46
TOTAL		4.72	26.00	

NO. OF ANIMALS EQUALS 9
NO. OF CONTAMINATED EQUALS 1

MEAN C/MEAN B = 5.51

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.52	2.89	5.85
RANGE	.59	4.00	8.53
MAX	.86	5.00	10.99
MIN	.27	1.00	2.46

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: INTERMEDIATE - 500 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JUNE 26, 1972

ANIMAL NUMBER	A RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5
1	463.00	.47	2.00	4.27
2	431.00	.43	2.00	4.64
3	443.00	.44	1.00	2.26
4	378.00	.38	3.00	7.94
5	309.00	.31	1.00	3.24
6	467.00	.47	2.00	4.28
7	317.00	.32	2.00	6.31
8	473.00	.47	4.00	8.46
9	478.00	.48	3.00	6.28
TOTAL		3.76	20.00	

NO. OF ANIMALS EQUALS 9
NO. OF CONTAMINATED EQUALS 1

MEAN C/MEAN B = 5.31

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.42	2.22	5.30
RANGE	.17	3.00	6.20
MAX	.48	4.00	8.46
MIN	.31	1.00	2.26

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JUNE 26, 1972

ANIMAL NUMBER	A RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5	
1	382.00	.38	2.00	5.24	
2	233.00	.23	1.00	4.29	
3	650.00	.65	7.00	10.77	
4	530.00	.53	4.00	7.55	
5	259.00	.26	5.00	19.31	
6	286.00	.29	8.00	27.97	*
7	623.00	.62	11.00	17.66	
8	342.00	.34	3.00	8.77	
9	297.00	.30	1.00	3.37	
TOTAL		3.60	42.00		

NO. OF ANIMALS EQUALS 9
NO. OF CONTAMINATED EQUALS 1

MEAN C/MEAN B = 11.66

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.40	4.67	11.66
RANGE	.42	10.00	24.61
MAX	.65	11.00	27.97
MIN	.23	1.00	3.37

* SUMMARY WITH OUTLIERS REMOVED

MEAN C/MEAN B = 10.25

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.41	4.25	9.62
RANGE	.42	10.00	15.94
MAX	.65	11.00	19.31
MIN	.23	1.00	3.37

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: NEGATIVE CONTROL - SALINE (SUBACUTE TRAILS)

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: JUNE 30, 1972

ANIMAL NUMBER	A RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5	
1	287.00	.29	.00	.00	*
2	471.00	.47	3.00	6.37	
3	392.00	.39	1.00	2.55	
4	280.00	.28	1.00	3.57	
5	571.00	.57	4.00	7.01	
6	300.00	.30	2.00	6.67	
7	243.00	.24	1.00	4.12	
8	399.00	.40	1.00	2.51	
9	428.00	.43	2.00	4.67	
10	463.00	.46	3.00	6.48	
TOTAL		3.33	18.00		

NO. OF ANIMALS EQUALS 10

MEAN C/MEAN B = 4.69

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.38	1.80	4.39
RANGE	.33	4.00	7.01
MAX	.57	4.00	7.01
MIN	.24	.00	.00

* SUMMARY WITH OUTLIERS REMOVED

MEAN C/MEAN B = 5.07

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.39	2.00	4.88
RANGE	.33	3.00	4.50
MAX	.57	4.00	7.01
MIN	.24	1.00	2.51

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: LOW - 50 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: JUNE 30, 1972

ANIMAL NUMBER	A RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5
1	470.00	.47	1.00	2.13
2	607.00	.61	2.00	3.29
3	513.00	.51	2.00	3.90
4	600.00	.60	3.00	5.00
5	328.00	.33	1.00	3.05
6	454.00	.45	1.00	2.20
7	490.00	.49	2.00	4.08
8	712.00	.71	4.00	5.62
9	380.00	.38	1.00	2.63
10	422.00	.42	1.00	2.37
TOTAL		4.98	18.00	

NO. OF ANIMALS EQUALS 10

MEAN C/MEAN B = 3.62

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.50	1.80	3.43
RANGE	.38	3.00	3.49
MAX	.71	4.00	5.62
MIN	.33	1.00	2.13

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-35 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: INTERMEDIATE - 500 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: JUNE 30, 1972

ANIMAL NUMBER	A RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5
1	742.00	.74	2.00	2.70
2	207.00	.21	1.00	4.83
3	566.00	.57	2.00	3.53
4	811.00	.81	2.00	2.47
5	227.00	.23	1.00	4.41
6	401.00	.40	2.00	4.99
7	203.00	.20	1.00	4.93
8	323.00	.32	3.00	9.29
9	372.00	.37	1.00	2.69
10	419.00	.42	3.00	7.16
TOTAL		4.27	18.00	

NO. OF ANIMALS EQUALS 10

MEAN C/MEAN B = 4.21

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.43	1.80	4.70
RANGE	.61	2.00	6.82
MAX	.81	3.00	9.29
MIN	.20	1.00	2.47

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-55 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: JUNE 30, 1972

ANIMAL NUMBER	A RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5
1	1000.00	1.00	3.00	3.00
2	631.00	.63	1.00	1.58
3	429.00	.43	1.00	2.33
4	423.00	.42	1.00	2.36
5	224.00	.22	1.00	4.46
6	481.00	.48	4.00	8.32
7	257.00	.26	1.00	3.89
8	807.00	.81	2.00	2.48
9	461.00	.46	3.00	6.51
10	477.00	.48	3.00	6.29
TOTAL		5.19	20.00	

NO. OF ANIMALS EQUALS 10

MEAN C/MEAN B = 3.85

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.52	2.00	4.12
RANGE	.78	3.00	6.73
MAX	1.00	4.00	8.32
MIN	.22	1.00	1.58

NO OUTLIERS

3. Cytogenetics

a. In Vivo

The chromosomal abnormalities observed in the positive controls were significantly higher than either the negative controls or the compound. The maximum effect of the positive control was observed at 48 hours after administering the compound. A depression of the mitotic index was observed in the positive control animals and in the subacute doses. The percentage of breaks produced by the compound in both the acute and subacute studies was higher than the negative control values at all dosage levels, except the usage subacute and the intermediate acute. The frequency of breaks in the negative controls was well with the range that we have seen in the past.

b. In Vitro

Anaphase preparations were examined in this test. The positive control compound produced a significantly higher percentage of aberrations in the chromosomes than the negative control or the test compound. Depression of the mitotic index due to the positive control compound was not as pronounced as in the in vivo test. At the high dosage level, 6% of the cells contained aberrations as compared to 2% of the negative controls. Negative controls were well within normal limits.

c. CYTOGENETICS SUMMARY SHEETS

FDA CONTRACT 71-268

FDA COMPOUND 71-35

SODIUM THIOSULFATE

SODIUM THIOSULFATE
FDA 71-35
ACUTE STUDY
METAPHASE SUMMARY SHEET

Compound	Dosage (mg/kg)	Time*	No. of Animals	No. of Cells	Mitotic*** Index %	% Cells with Breaks	% Cells with Reunion	% Cells Other Aber.**	% Cells with Aber. ++
Negative Control	Saline	6	3	150	8	0	0	0	0
		24	3	150	8	0	0	0	0
		48	3	150	9	3	0	0	3
Low Level	50	6	5	250	11	3	0	0	3
		24	5	250	8	6	0	0	6
		48	5	250	7	4	0	0	4
Intermediate Level	500	6	5	250	10	2	0	0	2
		24	5	250	10	2	0	0	2
		48	5	250	8	1	0	0	1
LD_5	5000	6	5	250	12	4	0	0	4
		24	5	250	7	5	0	0	5
		48	5	250	9	6	0	0	6
Positive Control TEM	0.3	48	5	250	4	22	18	9a	49

*Time of sacrifice after injection (hours)
**Cells that have polyploidy (P), pulverization (pp), or greater than 10 aberrations (a).
*** % of cells in mitosis:500 cells observed/animal.
++Duplicate aberrations in a single cell will cause this to be a % less than a summation of the % aberration seen.

SODIUM THIOSULFATE
FDA 71-35
SUBACUTE STUDY
METAPHASE SUMMARY SHEET

Compound	Dosage* (mg/kg)	No. of Animals	No. of Cells	Mitotic*** Index %	% Cells with Breaks	% Cells with Reunion	% Cells Other Aber.**	% Cells with Aber.
Negative Control	Saline	3	150	12	2	0	0	2
Low Level	50	5	250	6	4	0	0	4
Intermediate Level	500	5	250	5	7	0	0	7
LD_5	5000	5	250	6	6	0	0	6

*Dosage 1X/day X 5 days
**Cells that have polyploidy (P), pulverization (pp), or greater than 10 aberrations (a).
*** % of cells in mitosis:500 cells observed/animal.

SODIUM THIOSULFATE

FDA 71-35

ANAPHASE SUMMARY SHEET

Compound	Dosage (mcg/ml)	Mitotic** Index	No. of Cells	% Cells with Acentric Frag.	% Cells with Bridges	% Multipolar Cells	% Cells Other Aber.*	% Cells with Aber.++
Low Level	8.0	4	100	0	0	0	0	0
Medium Level	80	3	100	0	1	0	0	1
High Level	800	3	100	4	2	0	0	6
Negative Control	saline	6	100	2	0	0	0	2
Positive Control (TEM)	0.1	2	100	12	6	0	2	20

*Cells that have polyploidy (P), pulverization (pp), or greater than 10 aberrations (a).
** % of cells in mitosis:200 cells observed/animal.
++Duplicate aberrations in a single cell will cause this to be a % less than a summation of the % aberration seen.

4. Dominant Lethal Study

The interpretation of these data was made by Dr. David Brusick, Assistant Professor of Microbiology, Howard University, Washington, D. C. as a consultant to LBI.

Fertility Index

Acute - Significant dose-related decrease at the low dose in week 7.

Subacute - Significant increases at the intermediate dose in week 3.

Average # Implants/Pregnant Female

Acute - Significant dose-related decreases at the high dose of week 1 and the low dose of week 3. A significant decrease is also seen at the intermediate dose of week 4.

Subacute - Significant increase in week 7 at the low dose.

Average # Corpora Lutea/Pregnant Female

Acute - A significant dose-related increase was observed in week 3 at the high dose. Week 4 showed a significant increase at the low dose.

Subacute - A significant dose-related decrease was observed at the high dose of week 1. Significant increases were observed at the low doses of week 3&7 and at the intermediate dose of week 3. A significant dose-related increase was observed at all three doses in week 4.

Average Pre-implantation Losses/Pregnant Female

Acute - Significant increases were obtained at all three doses of week 3 and at the low and intermediate doses of week 4.

Subacute - Significant dose-related increases were obtained at all three dose levels of weeks 3 and 4. A significant decrease was observed at the low dose of week 6.

*Week 4 shows a consistent decrease trend in the subacute dose levels of the last four categories. This trend is also found in Compound #39 at the same doses.

c. DOMINANT LETHAL SUMMARY TABLES

FDA CONTRACT 71-268

FDA COMPOUND 71-35

SODIUM THIOSULFATE

All female rats in the dominant lethal assay were given tetracycline hydrochloride for the duration of the study. All male rats were given tetracycline hydrochloride beginning October 24, 1972 until November 7, 1972, started again November 15, 1972 and continued to the end of the study.

The daily dosage level contained in the cage water bottle was approximately 75 mg/kg.

TABLE I

COMPOUND 35 STUDY ACUTE

FERTILITY INDEX

OG ARITH OSE DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
	1	109/159=0.69	14/19=0.74	13/20=0.65	13/20=0.65	16/19=0.85	15/20=0.75
	2	119/159=0.75	17/20=0.85	16/19=0.85	13/20=0.65	16/20=0.80	16/20=0.80
	3	119/158=0.76	13/18=0.73	17/20=0.85	15/18=0.84	18/20=0.90	15/20=0.75
	4	136/160=0.85	19/20=0.95	18/19=0.95	16/20=0.80	17/20=0.85	14/18=0.78
	5	127/159=0.80	9/19=0.48 **	6/18=0.34 **	5/20=0.25 **	13/20=0.65	4/14=0.29 **
	6	128/159=0.81	17/20=0.85	17/19=0.90	15/20=0.75	16/20=0.80	15/20=0.75
	7	133/157=0.85	16/18=0.89	11/19=0.58* **	16/20=0.80	14/20=0.70	16/20=0.80
! !	8	133/160=0.84	16/20=0.80	14/19=0.74	11/20=0.55 **	14/20=0.70	17/20=0.85

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE II

COMPOUND 35 STUDY ACUTE

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
!	!	1	1351/109=12.4	180/14=12.9	158/13=12.2	151/13=11.6	179/16=11.2@D	162/15=10.8*@D
!	!						@D	*@D
		2	1427/119=12.0	214/17=12.6	198/16=12.4	179/13=13.8	206/16=12.9	146/16= 9.1**@@D
!						**@@I		**@@D
	!	3	1435/119=12.1	156/13=12.0	181/17=10.7@D	176/15=11.7	224/18=12.4	147/15= 9.8**@@D
					*@D			**@@D
		4	1626/136=12.0	232/19=12.2	222/18=12.3	178/16=11.1@D	201/17=11.8	157/14=11.2
						@D		
		5	1466/127=11.5	99/ 9=11.0	59/ 6= 9.8	56/ 5=11.2	132/13=10.2	46/ 4=11.5
!					*@D		@D	
		6	1512/128=11.8	200/17=11.8	197/17=11.6	184/15=12.3	178/16=11.1	162/15=10.8
								@D
		7	1626/133=12.2	192/16=12.0	142/11=12.9	189/16=11.8	163/14=11.6	175/16=10.9
								*@D
		8	1551/133=11.7	177/16=11.1	172/14=12.3	129/11=11.7	160/14=11.4	180/17=10.6

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
! AND @ = ONE-TAILED TEST

ONE !,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE III

COMPOUND 35 STUDY ACUTE

AVERAGE CORPORA LUTEA PER PREGNANT FEMALE

OG ARITH DSE DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
	1	1504/109=13.8	219/14=15.6 @I	206/13=15.9 *@@I	184/13=14.2	226/16=14.1	243/15=16.2 **@@I
	2	1588/119=13.3	250/17=14.7 *@@I	223/16=13.9	193/13=14.9 **@@I	226/16=14.1	232/16=14.5
!	3	1565/119=13.2	176/13=13.5	256/17=15.1 *@I	217/15=14.5 *@I	280/18=15.6*@I **@@I	192/15=12.8
&!! &&!!	4	1784/136=13.1	242/19=12.7	272/18=15.1*@@I *&@I	208/16=13.0	224/17=13.2	180/14=12.9
	5	1648/127=13.0	108/ 9=12.0 @D	60/ 6=10.0@D **@@D	63/ 5=12.6	142/13=10.9 **@@D	54/ 4=13.5*@I
&!! & !!	6	1689/128=13.2	227/17=13.4	213/17=12.5	196/15=13.1	201/16=12.6	200/15=13.3
	7	1767/133=13.3	212/16=13.3	149/11=13.6	205/16=12.8	174/14=12.4 @D	212/16=13.3
	8	1823/133=13.7	229/16=14.3	219/14=15.6 *@@I	144/11=13.1	195/14=13.9	242/17=14.2

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
! AND @ = ONE-TAILED TEST

ONE !,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE IV

COMPOUND 35 STUDY ACUTE

AVERAGE PREIMPLANTATION LOSSES PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
&!! & !		1	153/109= 1.4	39/14= 2.8 @I	48/13= 3.7 **@@I	33/13= 2.5 **@@I	47/16= 2.9 *@I	81/15= 5.4*@@I **@@I
		2	161/119= 1.4	36/17= 2.1	25/16= 1.6	14/13= 1.1	20/16= 1.3	86/16= 5.4**@@I **@@I
&!! &&!!		3	130/119= 1.1	20/13= 1.5	75/17= 4.4**@@I **@@I	41/15= 2.7@I **@@I	56/18= 3.1@I **@@I	45/15= 3.0@I **@@I
!		4	158/136= 1.2	10/19= 0.5	50/18= 2.8**@@I *@I	30/16= 1.9*@@I @I	23/17= 1.4	23/14= 1.6*@@I @I
		5	182/127= 1.4	9/ 9= 1.0	1/ 6= 0.2 **@@D	7/ 5= 1.4	10/13= 0.8	8/ 4= 2.0
		6	177/128= 1.4	27/17= 1.6	16/17= 0.9	12/15= 0.8	23/16= 1.4	38/15= 2.5
		7	141/133= 1.1	20/16= 1.3	7/11= 0.6	16/16= 1.0	11/14= 0.8	37/16= 2.3 **@@I
		8	272/133= 2.1	52/16= 3.3 @I	47/14= 3.4 **@@I	15/11= 1.4	35/14= 2.5	62/17= 3.7 *@I

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
! AND @ = ONE-TAILED TEST

ONE !,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE V

COMPOUND 35 STUDY ACUTE

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE

OG OSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTRCL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
!	& !	1	28/109=0.26	0/14=0.0 **@@D	2/13=0.16	1/13=0.08 @D	7/16=0.44@I	35/15=2.34**@@I **@@I
!		2	53/119=0.45	4/17=0.24	19/16=1.19*@I @I	10/13=0.77*@I @I	12/16=0.75	36/16=2.25**@@I **@@I
!		3	61/119=0.52	1/13=0.08 **@@D	2/17=0.12 **@@D	4/15=0.27	5/18=0.28	61/15=4.07**@@I **@@I
!		4	62/136=0.46	3/19=0.16 *@@D	5/18=0.28	1/16=0.07 **@@D	5/17=0.30	62/14=4.43**@@I **@@I
!		5	74/127=0.59	8/ 9=0.89	0/ 6=0.0 *@@D **@@D	0/ 5=0.0 *@@D **@@D	4/13=0.31	4/ 4=1.00
! !		6	58/128=0.46	4/17=0.24	9/17=0.53	16/15=1.07**@@I *@@I	11/16=0.69	18/15=1.20@I
		7	65/133=0.49	10/16=0.63	5/11=0.46	9/16=0.57	6/14=0.43	24/16=1.50
		8	71/133=0.54	13/16=0.82	6/14=0.43	6/11=0.55	10/14=0.72	18/17=1.06 **@@I

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTRCL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
! AND @ = ONE-TAILED TEST

ONE !,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VI

COMPOUND 35 STUDY ACUTE

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS

OG ARITH OSE DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
	1	24/109=0.23	0/14=0.0	2/13=0.16	1/13=0.08	4/16=0.25*	11/15=0.74** **
	2	38/119=0.32	4/17=0.24	9/16=0.57	8/13=0.62* *	7/16=0.44	11/16=0.69** **
	3	39/119=0.33	1/13=0.08	2/17=0.12	1/15=0.07 *	4/18=0.23	11/15=0.74** **
	4	46/136=0.34	3/19=0.16	2/18=0.12	1/16=0.07 *	4/17=0.24	12/14=0.86** **
	5	45/127=0.36	5/ 9=0.56	0/ 6=0.0 *	0/ 5=0.0 *	3/13=0.24	3/ 4=0.75
	6	44/128=0.35	3/17=0.18	7/17=0.42	10/15=0.67** *	6/16=0.38	7/15=0.47
	7	46/133=0.35	8/16=0.50	4/11=0.37	7/16=0.44	6/14=0.43	7/16=0.44
	8	50/133=0.38	7/16=0.44	4/14=0.29	5/11=0.46	7/14=0.50	12/17=0.71 **

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VII

COMPOUND 35 STUDY ACUTE

PORPORTION OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
		1	3/109=0.03	0/14=0.0	0/13=0.0	0/13=0.0	1/16=0.07	9/15=0.60** **
		2	14/119=0.12	0/17=0.0	3/16=0.19	1/13=0.08	4/16=0.25*	8/16=0.50** **
		3	17/119=0.15	0/13=0.0	0/17=0.0	1/15=0.07	1/18=0.06	11/15=0.74** **
		4	12/136=0.09	0/19=0.0	2/18=0.12	0/16=0.0	1/17=0.06	9/14=0.65** **
		5	18/127=0.15	1/ 9=0.12	0/ 6=0.0	0/ 5=0.0	1/13=0.08	1/ 4=0.25
		6	13/128=0.11	1/17=0.06	2/17=0.12	4/15=0.27	4/16=0.25	4/15=0.27
		7	14/133=0.11	2/16=0.13	1/11=0.10	1/16=0.07	0/14=0.0	3/16=0.19
		8	18/133=0.14	6/16=0.38 *	2/14=0.15	1/11=0.10	1/14=0.08*	6/17=0.36 *

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VIII

COMPOUND 35 STUDY ACUTE

DEAD IMPLANTS / TOTAL IMPLANTS

WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
1	28/1351=0.03	0/180=0.0 **@@D	2/158=0.02	1/151=0.01	7/179=0.04@I	35/162=0.22**@@I **@@I
2	53/1427=0.04	4/214=0.02	19/198=0.10	10/179=0.06	12/206=0.06	36/146=0.25**@@I **@@I
3	61/1435=0.05	1/156=0.01 **@@D	2/181=0.02 @D	4/176=0.03	5/224=0.03	61/147=0.42**@@I **@@I
4	62/1626=0.04	3/232=0.02 *@@D	5/222=0.03	1/178=0.01 **@@D	5/201=0.03	62/157=0.40**@@I **@@I
5	74/1466=0.06	8/ 99=0.09	0/ 59=0.0 @D **@@D	0/ 56=0.0 @D **@@D	4/132=0.04	4/ 46=0.09
6	58/1512=0.04	4/200=0.02	9/197=0.05	16/184=0.09*@I @I	11/178=0.07	18/162=0.12@I @I
7	65/1626=0.04	10/192=0.06	5/142=0.04	9/189=0.05	6/163=0.04	24/175=0.14
8	71/1551=0.05	13/177=0.08	6/172=0.04	6/129=0.05	10/160=0.07	18/180=0.10 **@@I

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT DIFFERENCES USING
THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT DIFFERENCES USING
THE HISTORICAL CONTROL GROUP

* = TWO-TAILED TEST
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TWO *,@ = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL

TABLE I

COMPOUND 35 STUDY SUBACUTE

FERTILITY INDEX

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
		1	104/159=0.66	11/20=0.55	15/19=0.79	15/18=0.84	16/20=0.80
		2	118/160=0.74	15/20=0.75	14/20=0.70	18/20=0.90	14/18=0.78
		3	119/159=0.75	14/20=0.70	14/20=0.70	19/20=0.95* *	17/19=0.90
		4	120/154=0.78	12/15=0.80	17/20=0.85	18/20=0.90	17/20=0.85
		5	122/157=0.78 :	11/19=0.58	8/19=0.43 **	11/20=0.55 *	14/19=0.74
		6	136/159=0.86	16/20=0.80	18/20=0.90	18/20=0.90	18/19=0.95
! !		7	135/155=0.88	13/18=0.73	15/20=0.75	15/20=0.75	15/20=0.75

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

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* SIGNIFICANTLY DIFFERENT FROM CONTROL
! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE II

COMPOUND 35 STUDY SUBACUTE

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
		1	1231/104=11.8	134/11=12.2	175/15=11.7	162/15=10.8	178/16=11.1
! & ! &!! &&!!		2	1474/118=12.5	180/15=12.0	175/14=12.5	202/18=11.2 *@D	142/14=10.1 *@D
&!! & !!		3	1405/119=11.8	155/14=11.1	159/14=11.4	200/19=10.5 *@@D	176/17=10.4 *@@D
		4	1414/120=11.8	135/12=11.3	197/17=11.6	213/18=11.8	207/17=12.2
		5	1462/122=12.0	127/11=11.6	90/ 8=11.3	135/11=12.3	161/14=11.5
		6	1626/136=12.0	187/16=11.7	217/18=12.1	212/18=11.8	202/18=11.2
		7	1566/135=11.6	140/13=10.8	185/15=12.3*@I @I	170/15=11.3	172/15=11.5

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

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&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

COMPOUND 35 TABLE III STUDY SUBACUTE

AVERAGE CORPORA LUTEA PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
& !	!	1	1385/104=13.3	184/11=16.7	236/15=15.7	235/15=15.7	231/16=14.4*@D
&&!!					**@@I	**@@I	**@@I *@I
	!	2	1599/118=13.6	208/15=13.9	201/14=14.4	256/18=14.2	178/14=12.7
		3	1535/119=12.9	169/14=12.1	189/14=13.5@I	258/19=13.6*@I	224/17=13.2
& !		4	1499/120=12.5	144/12=12.0	234/17=13.8@I	248/18=13.8*@I	237/17=13.9*@I
&&!! & !					@I	*@@I	*@I
		5	1554/122=12.7	137/11=12.5	94/ 8=11.8	140/11=12.7	170/14=12.1
		6	1809/136=13.3	214/16=13.4	223/18=12.4	239/18=13.3	239/18=13.3
					@D		
		7	1711/135=12.7	159/13=12.2	208/15=13.9@I	192/15=12.8	193/15=12.9
					*@I		

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

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&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE IV

EXPERIMENT 35 STUDY SUBACUTE

AVERAGE PREIMPLANTATION LOSSES PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
		1	154/104= 1.5	50/11= 4.6	61/15= 4.1	73/15= 4.9	53/16= 3.3
&!!	& !			**@@I	**@@I	**@@I	**@@I
		2	125/118= 1.1	28/15= 1.9	26/14= 1.9	54/18= 3.0	36/14= 2.6
&!!	& !!					**@@I	*@I
&!!		3	130/119= 1.1	14/14= 1.0	30/14= 2.1*@@I	58/19= 3.1*@@I	48/17= 2.8**@@I
&!!	&&!!				**@@I	**@@I	**@@I
!		4	85/120= 0.7	9/12= 0.8	37/17= 2.2@I	35/18= 1.9*@@I	30/17= 1.8*@I
&!!	&&!!				**@@I	**@@I	**@@I
		5	92/122= 0.8	10/11= 0.9	4/ 8= 0.5	5/11= 0.5	9/14= 0.6
		6	183/136= 1.4	27/16= 1.7	6/13= 0.3**@@D	27/18= 1.5	37/19= 2.1
					**@@D		
		7	145/135= 1.1	19/13= 1.5	23/15= 1.5	22/15= 1.5	21/15= 1.4

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

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*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE V

COMPOUND 25 STUDY SUBACUTE

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE

LOG OSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
	& !	1	40/104=0.39	2/11=0.19	4/15=0.27	0/15=0.0 **@@D	10/16=0.63
		2	59/118=0.50	2/15=0.14 *@@D	2/14=0.15 *@D	26/18=1.45	4/14=0.29
		3	69/119=0.58	6/14=0.43	3/14=0.22 @D	9/19=0.48	8/17=0.48
&!!	& !!	4	66/120=0.55	20/12=1.67 *@I	4/17=0.24 *@@D @D	1/18=0.06 **@@D **@@D	0/17=0.0 **@@D **@@D
&!!	&& !!	5	78/122=0.64	6/11=0.55	1/ 8=0.13 **@@D	3/11=0.28 @D	3/14=0.22 *@@D
!		6	62/136=0.46	9/16=0.57	6/18=0.34	19/18=1.06 *@@I	13/18=0.73
		7	70/135=0.52	6/13=0.47	6/15=0.40	6/15=0.40	4/15=0.27

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
! AND @ = ONE-TAILED TEST

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TWO !,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VI

COMPOUND 3F STUDY SUBACUTE

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
		1	31/104=0.30	1/11=0.10	2/15=0.14	0/15=0.0 *	5/16=0.32
		2	38/118=0.33	2/15=0.14	2/14=0.15	4/18=0.23	4/14=0.29
		3	42/119=0.36	2/14=0.15	3/14=0.22	5/19=0.27	5/17=0.30
!!	!!	4	42/120=0.35	8/12=0.67 *	4/17=0.24*	1/18=0.06** *	0/17=0.0 ** **
!!	!	5	54/122=0.45	3/11=0.28	1/ 8=0.13	2/11=0.19	2/14=0.15 *
!		6	43/136=0.32	7/16=0.44	6/18=0.34	11/18=0.62 *	8/18=0.45
		7	42/135=0.32	5/13=0.39	6/15=0.40	5/15=0.34	3/15=0.20

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VII

COMPOUND 35 STUDY SUBACUTE

PORPORTION OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
		1	8/104=0.08	1/11=0.10	2/15=0.14	0/15=0.0	3/16=0.19
		2	10/118=0.09	0/15=0.0	0/14=0.0	2/18=0.12	0/14=0.0
		3	17/119=0.15	1/14=0.08	0/14=0.0	2/19=0.11	2/17=0.12
!		4	15/120=0.13	4/12=0.34	0/17=0.0 *	0/18=0.0 **	0/17=0.0 *
!				*			
		5	19/122=0.16	2/11=0.19	0/ 8=0.0	1/11=0.10	1/14=0.08
		6	13/136=0.10	2/16=0.13	0/18=0.0	6/18=0.34	5/18=0.28
						**	*
		7	16/135=0.12	1/13=0.08	0/15=0.0	1/15=0.07	1/15=0.07

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,* = SIGNIFICANT AT P LESS THAN 0.01

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! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VIII

COMPOUND 35 STUDY SUBACUTE

DEAD IMPLANTS / TOTAL IMPLANTS

WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 50.000 MG/KG	DOSE LEVEL 500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
1	40/1231=0.04	2/134=0.02	4/175=0.03	0/162=0.0 *@@D	10/178=0.06
2	59/1474=0.05	2/180=0.02 **@@D	2/175=0.02 **@@D	26/202=0.13	4/142=0.03@I
3	69/1405=0.05	6/155=0.04	3/159=0.02 *@@D	9/200=0.05	8/176=0.05
4	66/1414=0.05	20/135=0.15 @I	4/197=0.03*@D @D	1/213=0.01*@D **@@D	0/207=0.0 *@@D **@@D
5	78/1462=0.06	6/127=0.05	1/ 90=0.02 **@@D	3/135=0.03 @D	3/161=0.02 *@D
6	62/1626=0.04	9/167=0.05	6/217=0.03	19/212=0.09@I *@I	13/202=0.07
7	70/1566=0.05	6/140=0.05	6/185=0.04	6/170=0.04	4/172=0.03

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT DIFFERENCES USING
THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT DIFFERENCES USING
THE HISTORICAL CONTROL GROUP

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ONE *,@ = SIGNIFICANT AT P LESS THAN 0.05
TWO *,@ = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL

II. APPENDICES (MATERIALS AND METHODS)

A. Animal Husbandry

1. Animals--Rats and Mice

Ten to twelve week old rats (280 to 350 g) and male mice (25-30 g) were fed a commercial 4% fat diet and water ad libitum until they were on experiment. Flow Laboratories random-bred, closed colony, Sprague-Dawley CD strain rats were used in the cytogenetic studies. Flow Laboratories ICR male mice were employed in the Host-Mediated Assay.

2. Preparation of Diet

A commercial 4% fat diet was fed to all animals. Periodic tests to verify the absence of coliforms, Salmonella and Pseudomonas sp. were performed.

3. Husbandry

Animals were held in quarantine for 4-11 days. Mice were housed five to a cage and rats one to five to a cage. Animals were identified by ear punch. Sanitary cages and bedding were used, and changed two times per week, at which time water containers were cleaned, sanitized and filled. Once a week, cages were repositioned on racks; racks were repositioned within rooms monthly. Personnel handling animals or working with animal facilities wore head covering and face masks, as well as suitable garments. Individuals with respiratory or other overt infections were excluded from the animal facilities.

B. Dosage Determination

1. Acute LD_{50} and LD_5 Determination

Since the compounds proposed for testing are included in the

food additive regulations as "generally recognized as safe" (GRAS), it was expected that a large number of them would be sufficiently non-toxic so that determination of an LD_{50} or an LD_5 is of no practical value. In fact, this has been our experience with previously tested compounds from this list. In the case of these relatively non-toxic compounds, attempts were made to assure that the amounts to be administered would not affect the animals by means (mechanical, physical, etc.) related to their bulk rather than to their toxicity. In the cases of certain compounds where an LD_{50} or an LD_5 were not determined, an exceedingly high concentration, 5 g/kg, was employed and accepted as the LD_5 level. In cases where the toxicity was high enough to allow determination of an LD_{50}, the following protocol was used.

Thirty rats of the strain chosen for studies described below and of approximately the age and weight specified were assigned at random to six groups. Each group was then given, using the chosen route of administration, one of a series of dosages of the test compound following a logarithmic dosage scheme. The series of dosages was derived from a consideration of whatever toxicity information was available for the particular test compound. The objective in selecting dosages was to choose values which would cause mortalities between 10% and 90%.

When information was inadequate to derive a suitable series of dosages, five rats were used to identify the proper range. Each of these was given one of a widely spaced (differing by 10X) series of doses. This was confidently expected to suffice for derivation of the series of dosages to be used in the LD_{50} determination.

The mortalities observed when the series of dosages was given to the 30 rats were then subjected to a probit analysis and calculation of LD_{50}, LD_5, slope and confidence limits by the method of Litchfield and

Wilcoxon. The highest dose level used was either a finite LD_5 or 5000mg/kg. The intermediate level used was either 1/10 of the finite LD_5 or 500mg/kg. The low level used was either 1/100 of the finite LD_5 or 50 mg/kg.

2. Subacute Studies

Subacute doses were identical to those used in the acute studies. Each subacute study animal was given the acute dosage once a day for each of five consecutive days (24 hours apart).

C. Mutagenicity Testing Protocols

1. Host Mediated Assay

Flow Laboratories ICR random-bred male mice were used in this study. In the acute and subacute studies ten animals, 25-30 g each, were employed at each dose level. Solvent and positive controls were run at all times. The positive control (Dimethyl nitrosamine) was run by the acute system only at a dose of 100 mg/kg for Salmonella. For yeast, ethyl methane sulfonate (EMS) intramuscularly injected at a dose of 350 mg/kg was used. The solvents used and the toxicity data are presented in the Results and Discussion section of the report.

The indicator organisms used in this study were: (1) two histidine auxotrophs [his G-46, TA-1530] of Salmonella typhimurium, and (2) a diploid strain [D-3] of Saccharomyces cerevisiae. The induction of reverse mutation was determined with the Salmonella; mitotic recombination was determined with yeast. Chemicals were evaluated directly by in vitro bacterial and yeast studies prior to, or concurrent with, the studies in mice. Animals on acute studies only were not fed the evening prior to compound administration. The Salmonella were carried in tryptone yeast extract gel, transferred weekly. They were transferred to tryptone yeast extract broth 48 hours

before use: they were transferred a second time from broth to broth 24 hours prior to use, and again 8 hours before use. The mouse inoculum was prepared by transferring 4 ml of the 8-hour broth culture to 50 ml broth bottles which had been prewarmed to 37°C. Exponential log-phase organisms were inoculated intraperitoneally into the mice approximately 2-1/2 hours later when the appropriate density indicating 3.0×10^8 cells/ml was reached. The Saccharomyces was carried in yeast complete agar. The inoculum was prepared by harvesting the organisms from the surface of the plates with sterile saline. The cells were washed three times with sterile saline and suspended in a concentration of 5.0×10^8 cells/ml. Two ml of the suspension was inoculated into each mouse intraperitoneally. Total plate counts on Salmonella were on tryptone yeast extract and for Saccharomyces on yeast complete medium.

a. Acute Study

Three dosage levels (usage, intermediate [determined as discussed previously], and LD_5) were administered orally by intubation to ten mice. Positive controls and negative vehicle controls were included in each study. All animals received 2 ml of the indicator organism intraperitoneally. Each ml contained 3.0×10^8 cells for Salmonella and 5.0×10^6 cells for Saccharomyces. Three hours later, each animal was killed and 2 ml of sterile saline was introduced intraperitoneally. As much fluid as possible was then aseptically removed from the peritoneal cavity. Dilution blanks for bacteria containing 4.5 ml of sterile saline were prepared in advance. Tenfold serial dilutions were made of each peritoneal exudate (0.5 ml exudate + 4.5 ml saline)

yielding a concentration series from 10^0 (undiluted peritoneal exudate) through 10^{-7}. For enumeration of total bacterial counts, the 10^{-6} and 10^{-7} dilutions were plated on tryptone yeast extract agar, 3 plates/sample. 0.2 ml sample/plate. Each sample was spread over the surface of the plate using a bent glass rod immersed in 95% ethanol and flamed just prior to use. In plating for the total mutant counts on minimal agar, the 10^0 dilution was used, 0.2 ml being plated on each of 5 plates. The plating procedure was identical to that followed for the tryptone yeast extract agar plates. All plates were incubated at 37°C, tryptone yeast extract agar plates for 18 hours and minimal agar plates for 40 hours. For yeast mitotic recombination, dilution blanks containing 4.5 ml of sterile saline were prepared in advance. Ten-fold serial dilutions were made of each sample yielding a series from 10^0 to 10^{-5}. Samples of 0.1 ml of the 10^{-5}, 10^{-4}, and 10^{-3} dilutions were removed and plated on complete medium (10 plates each). All plates were incubated at 30°C for 40 hours. The 10^{-5} dilutions were used to determine total populations and the 10^{-4} and 10^{-3} plates were examined after an additional 40 hours at 4°C for red sectors indicating a mutation. Bacterial scoring was calculated as follows:

Total mutants on 5 plates X appropriate exponent = CFU/ml of sample plated

(CFU is Colony Forming Units)

CFU/ml X one/dilution factor (10^0 - 10^{-7}) = CFU/ml in undiluted exudate.

The mutation frequency (MF) was calculated for each sample where:

$$MF = \frac{\text{total mutant cells}}{\text{total population}}$$

$$MFt/MFc = \frac{\text{MF of experimental sample}}{\text{MF of control sample}}$$

(MFt/MFc = 1.00 for control sample)

Yeast mitotic recombinants (presumptive ade 2, his 8 homozygotes) were seen as red colonies or as red sectors on a normally white yeast colony. The plates (from 10^{-4} and 10^{-3} dilutions) were scanned under the 10X lens of a dissecting scope to enumerate the red colonies and sectors. Population determinations were made from the 10^{-5} dilution plates. A recombinant frequency (RF) was calculated:

$$RF = \frac{\text{total recombinants counted}}{\text{total number colonies screened}}$$

b. Subacute Study

Similar groups of animals at each dose level received five oral doses of the test compound 24 hours apart. Within 30 minutes after the last dosing, the animals were inoculated with the test organism and handled in the same fashion as those in the acute study.

c. In Vitro Study

Cultures of S. typhimurium histidine auxotrophs (G-46 and TA-1530) were plated on appropriate media. The test compound was then added to the plate, either in the form of a microdrop of solution (0.01 to 0.25 ml) applied to a small filter paper disc resting on the agar or a small crystal applied directly to the agar. Tenfold serial dilutions of the culture were employed and plated so as not to miss the optimum cell density for mutant growth. Mutant colonies were observed and scored. Strain D-3 Saccharomyces cells at proper dilutions were shaken with the test compound, diluted, and plated at 50% survival level or above (see HMA Supplementary Materials and Methods). Red sectors were then scored and the frequency calculated after suitable incubation. Negative and positive controls were run concurrently. The positive control was EMS for Salmonella and Saccharomyces. The in vitro Salmonella tests were reported as (+) or (-) or questionable; the in vitro Saccharomyces tests were reported as sample concentrations, percent survival,

and recombinants/10^5 survivors. For the Saccharomyces a 50% survival level, e.g., an arbitrary 5.0% w/v test level, was used when no LD_{50} was determinable.

2. Cytogenetic Studies

a. In Vivo Study

Ten to twelve week old, male, albino rats obtained from a closed colony (random-bred) were used. A total of 59 animals in the acute study and 18 animals in the subacute study was used, as illustrated in the following protocol.

Number of Animals Used

Acute Study

Treatment	Time Killed after Administration		
	6 hours	24 hours	48 hours
High level	5	5	5
Intermediate level	5	5	5
Low level	5	5	5
Positive control	0	0	5
Negative control	3	3	3

Subacute Study

Five doses 24 hours apart; animals killed 6 hours after last dose.

Treatment	Killed after Administration
High level	5
Intermediate level	5
Low level	5
Negative control	3

All animals were dosed by gastric intubation.

Four hours after the last compound administration, and two hours prior to killing, each animal was given 4 mg/kg of colcemid intraperitoneally in

order to arrest the bone marrow cells in C-mitosis. Animals were killed by using CO_2, and the adhering muscle and epiphysis of one femur were removed. The marrow "plug" was removed with a tuberculin syringe and an 18 gauge needle, aspirated into 5 ml of Hanks' balanced salt solution (BSS) in a test tube and capped. The specimens were centrifuged at 1,500 rpm in a table-top centrifuge for 5 minutes, decanted, and 2 ml of hypotonic 0.5% KCl solution was added with gentle agitation to resuspend the cells. The specimens were then placed in a 37°C water bath for 20 minutes in order to swell the cells. Following centrifugation for 5 minutes at 1,500 rpm, the supernatant was decanted and 2 ml of fixative (3:1 absolute methanol: glacial acetic acid) was added. The cells were resuspended in the fixative with gentle agitation, capped, and placed at 4°C for 30 minutes. The specimens were again centrifuged, decanted, 2 ml of prepared fixative was added, and the cells were resuspended and placed at 4°C overnight.

The following day the specimens were again centrifuged, decanted and 0.3 - 0.6 ml of freshly prepared fixative was added to obtain a suitable density. The cells were resuspended and 2 - 3 drops of the suspension were allowed to drop onto a clean, dry slide held at 15° from the horizontal. As the suspension flowed to the edge of the slide, it was ignited by an alcohol burner and allowed to flame. Following ignition, the slides were allowed to dry at room temperature overnight. Duplicate slides were prepared. The slides were stained using a 5% Giemsa solution (Giemsa buffer pH 7.2) for 20 minutes, rinsed in acetone, 1:1 acetone:xylene, and placed in fresh xylene for 30 minutes. The slides were then mounted using permount (Fisher Scientific) and 24 X 50 mm coverglasses. The coverglasses were selected to be 0.17 mm $\pm$ 0.005 mm in thickness by use of a coverglass micrometer.

The preparations were examined using Leitz Ortholux I & II microscopes with brightfield optics and xenon light sources. These specimens were scanned with 10X and 24X objectives and suitable metaphase spreads that were countable were then examined critically using 40X, 63X or 100X oil immersion flat-field apochromatic objectives. Oculars were either 12X or 16X widefield periplanatics and the tube magnification either 1X or 1.25X. The filters used were either a didymium (BG20) or a Schott IL570 mμ interference filter.

The chromosomes for each cell were counted and only diploid cells were analyzed. They were scored for chromatid gaps and breaks, chromosome gaps and breaks, reunions, cells with greater than ten aberrations, polyploidy, pulverization, and any other chromosomal aberrations which were observed. They were recorded on the currently used forms and expressed as percentages on the summary sheets. Fifty metaphase spreads were scored per animal. Mitotic indices were obtained by counting at least 500 cells and the ratio of the number of cells in mitosis/the number of cells observed was expressed as the mitotic index.

Positive controls in the acute study consisted of animals which had been given the known mutagen Triethylene Melamine (TEM) administered intraperitoneally at a level of 0.30 mg/kg. Negative controls on the acute and subacute studies consisted of the vehicle in which the compound was administered. The dosage levels, solvents and toxicity data are included in the Results and Discussion section of the report.

b. In Vitro Study

Human embryonic lung cultures (WI-38) which were negative for adventitious agents (viruses, mycoplasma) which may interfere were used. These cells were employed at passage level 19. The cells had been transferred

using 0.025% trypsin and planted in 32 oz. prescription bottles containing 40 ml of tissue culture medium. When growth was approximately 95% confluent the cells were removed from the glass using trypsin, centrifuged, and frozen in tissue culture medium containing dimethyl sulfoxide (DMSO). Cells were frozen in vials in the vapor phase of liquid nitrogen at a concentration of 2×10^6 cells/ml. When needed, the vials were removed from liquid nitrogen, quick-thawed in a 37°C water bath, washed free of DMSO, suspended in tissue culture medium (minimal essential medium [MEM] plus 1% glutamine, 200 units/ml of penicillin and 200 µg/ml of streptomycin and 15% fetal calf serum) and planted in milk dilution bottles at a concentration of 5×10^5 cells/ml. The test compound was added at three dose levels using three bottles for each level, 24 hours after planting. The dose levels required a preliminary determination of a tissue culture toxicity. This was accomplished by adding logarithmic doses of the compound in saline to a series of tubes containing 5×10^5 cells/ml which were almost confluent. The cells were examined at 24, 48, and 72 hours. Any cytopathic effect (CPE) or inhibition of mitoses was scored as toxicity. Five more closely spaced dose levels were employed within the two logarithmic dosages, the higher of which showed toxicity and the lower no effect. The solvents used and the range finding data are presented in the toxicity data report under Results and Discussion. The dose level below the lowest toxic level was employed as the high level. Logarithmic dose levels were employed for the medium and low levels.

Cells were incubated at 37°C and examined twice daily to determine when an adequate number of mitoses were present. Cells were harvested by shaking when sufficient mitoses were observed, usually 24 - 48 hours after planting, centrifuged, and fixed in absolute methanol:glacial acetic acid (3:1) for 30 minutes.

The specimens were centrifuged, decanted, and suspended in acetic acid-orcein stain (2.0%) and a drop of suspension placed on a clean dry slide. Selected coverglasses 0.17 mm in thickness were placed on the suspension and the excess stain gently expressed from the slide. The coverglasses were sealed with clear nail polish and examined immediately.

The microscopes, objectives, oculars, filters and light sources were enumerated under the metaphase description. Positive controls used were TEM (at a concentration of 0.1 mcg/ml dissolved in saline) and negative controls which consisted of the vehicle in which the test compound was dissolved, which was 0.85% saline. Data were reported on forms currently used and expressed as percentages on the anaphase summary sheets.

3. Dominant Lethal Assay

In this test, male and female random bred rats from a closed colony were employed. These animals were 10-12 weeks old at the time of use. Ten male rats were assigned to each of 5 groups; 3 dose levels selected as described above, a positive control (triethylene melamine) (TEM) and a negative control (solvent only). The positive control was administered intraperitoneally. Administration of the test compound was orally by intubation in both the acute study (1 dose) and in the subacute study (1 dose per day for 5 days). Following treatment, the males were sequentially mated to 2 females per week for 8 weeks (7 weeks in the subacute study).

Two virgin female rats were housed with a male for 5 days (Monday through Friday). These two females were removed and housed in a cage until killed. The male was rested on Saturday and Sunday and two new females introduced to the cage on Monday. It has been our experience that conception has taken place in more than 90% of the females by Friday and that the two day rest is beneficial to the male as regards subsequent weekly matings. Females were killed using CO_2 at 14 days after separating from the male, and at necropsy the uterus was examined for deciduomata (early deaths), late fetal deaths and total implantations.

Sufficient animals were provided in our experimental design to accomodate for any reduction in the number of conceptions. Each male was mated with two females per week, and this provided for an adequate number of implantations per group per week (200 minimum) for negative controls, even if there was a four-fold reduction in fertility of implantations. Results were analyzed according to the statistical procedures described in Supplementary Materials and Methods. Corpora lutea, early fetal deaths, late fetal deaths and total implantations per uterine horn were recorded on the raw data sheets, which are submitted separately.

D. Supplementary Materials and Methods

1. Host Mediated Assay In Vitro and Formulae

a. Bacterial in vitro plate tests.

This method has been published by Ames: The Detection of Chemical Mutagens with Enteric Bacteria, in Chemical Mutagens; Principles and Methods for Their Detection, Vol. 1, Chapter 9, pp. 267-282, A Hollaender, Editor, Plenum Press, New York (1971).

b. In vitro for Mitotic Recombination.

(1) Strain D-3 was grown to stationary phase on complete medium agar plates at 30°C. (3-4 days). Cells were rinsed from the plates and washed twice in saline and cell concentration determined spectro-photometrically. (A standard curve previously determined for colony forming units versus % transmittance at 545 mu was easily used).

(2) Cells from the concentration suspension were diluted appropriately into 0.067 M Phosphate buffer pH 7.2 to provide 5×10^7 cells/ml in a total of 25 ml.

(3) The test chemical was first tested for 4 hours at 30°C, with shaking, at concentrations which permitted determination of the 50% survival level. Then, if not included in the first experiment, the compound was tested again only at the 50% survival level. If 50% survival level could not be determined, the arbitrary test level of 5% w/v was used.

(4) Following treatment, cells were diluted and plated on complete agar medium for determination of total population and red sectors. Total surviving population was conveniently measured on plates of 10^{-4} and 10^{-5} dilutions using 0.2 ml per plate (5 plates)

and sectors determined on plates of 10^{-3} and 10^{-4} dilutions using 0.2 ml per plate (5 plates). Plates were incubated for 2 days at 30°C followed by a holding period 2 days at 4°C to promote color development with limited enlargement of the colonies. Red sectors were scored by systematically scanning the plates with a dissecting microscope at 10X magnification.

(5) The frequency of red sectors was calculated and expressed as sectors per 10^5 survivors for comparison with untreated controls.

(6) Ethyl Methane Sulfonate (EMS) was employed as the positive control in both in vitro systems.

c. Minimal Medium (Bacteria):

Spizizen's Minimal Medium

4X Salt Solution:

$(NH_4)\ SO_4$	8.0	g
K_2HPO_4	56.0	g
KH_2PO_4	24.0	g
Na Citrate	4.0	g
$Mg\ SO_4$	0.8	g
Biotin	0.004	g
H_2O	qs to 1 liter	

Sterilize by autoclaving (121°C/15 min.)

Medium:

4X Salt Solution	250 ml	
5.0% Glucose (sterile)	100 ml	(If histidine is added at concentration of 30 mg/liter, this
1.5% Bacto-agar (sterile):	650 ml	becomes a complete bacterial medium.)

d. Complete medium (bacterial):

Bacto-tryptone	1.0 gm
Yeast-extract	0.5 gm
Bacto-agar	2.0 gm
Distilled H_2O	100.0 ml

Sterilize by autoclaving (121°C for 15 minutes)

e. Complete medium (yeast):

KH_2PO_4	1.5 gm
$MgSO_4$	0.5 gm
$(NH_4)_2SO_4$	4.5 gm
Peptone	3.5 gm
Yeast extract	5.0 gm
Glucose	20.0 gm
Agar	20.0 gm
Distilled H_2O	1000.0 ml

Sterilize by autoclaving (121°C for 15 minutes)

2. Cytogenetics In Vitro Preparation of Anaphase Chromosomes (from Nichols, 1970)

"Anaphase preparations may be made by several methods. One convenient approach is to grow cells directly on coverslips in petri dishes. With human fibroblasts 400,000 cells added to a 22 x 40 mm coverslip in a 50 mm petri dish grown in a 5% CO_2 atmosphere in air has proved very satisfactory. When adequate numbers of mitoses are visualized directly utilizing an inverted microscope (usually 48 to 92 hrs. after planting) the coverslip is transferred to absolute ethanol for 15 minutes for fixation. They are then stained with any one of a number of suitable stains (Fuelgen, May-Grunwald-Giemsa, orcein) and attached to a slide with mounting media for evaluation. Anaphase preparations may also be prepared on cells grown in suspension or cells from a monolayer that have been put into suspension. In this instance the cells are centrifuged and fixed with the squash fixative. They are then suspended in the stain and a drop of the suspension put on the slide and covered with a coverslip. However, in this case, only the excess stain is gently expressed from under the coverslip and no squashing is carried out. In anaphase preparations no pretreatment with colchicine or hypotonic expansion is used and no technique for spreading the cells is used, so that the spindle and normal relationships of the chromosomes are not disturbed."

3. Statistical Analyses of Dominant Lethal Studies

The following statistical analyses were employed as a means of analyzing the results of the dominant lethal studies.

a. The fertility index: number of pregnant females/number of mated females with the chi-square test used to compare each treatment to the control. Armitage's trend used for linear proportions to test whether the fertility index was linearly related to arithmetic or log dose.

b. Total number of implantations: t-test used to determine significant differences between average number of implantations per pregnant female for each treatment compared to the control. Regression techniques used to determine whether the average number of implantations per female was related to the arithmetic or log dose.

c. Total number of corpora lutea: t-test used to determine significant differences between average number of corpora lutea per pregnant female for each treatment compared to the control.

d. Preimplantation losses: computed for each female by subtracting number of implantations from number of corpora lutea. Freeman-Tukey transformation used on the preimplantation losses for each female and then t-test used to compare each treatment to control. Regression technique used to determine whether the average number of preimplantation losses per female was related to the arithmetic or log dose.

e. Dead implants: treated same as preimplantation losses.

f. The proportion of females with one or more dead implants computed, each treatment compared to control by chi-square test and Armitage's trend used for linear proportions to see if proportions were linearly related to either arithmetic or log dose. Also, probit regression analysis used to determine whether the probit of the proportions was related to log dose.

g. The proportion of females with two or more dead implants computed treated same as f.

h. Dead implants/total implants: computed for each female and used Freeman-Tukey arc-sine transformation on data for each female; then used t-test to compare each treatment to control.

Historical control data was compiled on a continuous basis as studies were completed. In addition to comparing each treatment to control, as outlined above, each treatment was compared to an historical control.

In order to take variation between males into account, a nested model was used. An analyses of across weeks is also provided.

In addition to these tests, the distribution forms of the various parameters were tested in order to evaluate the appropriateness of some of the tests being used. Certain correlations between parameters may exist and were examined as one step to determine the appropriateness of models. If necessary, alternate test methods were implemented.

The results are presented in tabular form with the addition of historical control information. In addition to these tables, a written report of all findings is provided. As information became available from the on-going investigation of these data, it was reported and suggestions included for changes to the methods of analyses. The statistical reports give the level of significance using both a one-tailed and two-tailed test. Finally, a summary sheet for each study is provided.

MODEL

$$y_{ijk} = \mu + \alpha_i + c_{ij} + e_{ijk},$$

$i = 1, 2$ Groups $\quad j = 1, 2, \ldots, 10$ Males within each group

$k = 1, 2$ Females within Males within Groups

ASSUMPTIONS: $\alpha_1 + \alpha_2 = 0$, $c_{ij} \sim nid(0, \sigma_c^2)$,

$e_{ijk} \sim nid(0, \sigma^2)$

Males are randomly drawn from infinite population

S.V.	d.f.	S.S.	M.S	E(M.S)	F
TOTAL	39	$\Sigma\Sigma\Sigma(y_{ijk} - \bar{y}_{...})^2$			
GROUPS	1	$20\Sigma(\bar{y}_{i..} - \bar{y}_{...})^2$	S_1^2	$\sigma^2 + 2\sigma_i^2 + 20\alpha_i^2$	
MALES WITHIN GROUPS	18	$2\Sigma\Sigma(\bar{y}_{ij.} - \bar{y}_{i..})^2$	S_2^2	$\sigma^2 + 2\sigma_c^2$	
REMAINDER	20	$\Sigma\Sigma\Sigma(y_{ijk} - \bar{y}_{ij.})^2$	S_3^2	σ^2	

E. References

Host-Mediated Assay

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F. Abbreviations

mu = micron

mcg = ug = microgram

g = gram

kg = kilogram

ml = milliliter

rpm = revolutions per minute

^{o}C = degrees centigrade

pH = power of the hydrogen ion concentration to the base 10

M = molar solution

conc. = concentration

MTD = maximum tolerated dosage = High = LD_5 if determined or else exceedingly high dose, such as 5 g/kg

INT = intermediate = medium level

USE = usage level if known = low level

BSS = balanced salt solution

C-metaphase = cells arrested in metaphase, using colchine or colcemid

LD_{50} = that dosage which produced 50% mortality in the group of animals treated

LD_5 = that dosage which produced 5% mortality in the group of animals treated

NC = negative control

PC = positive control

AU = acute usage level (low level)

AI = acute intermediate level (medium level)

AMTD = acute maximum tolerated dose level (LD_5 level, high level)

SAU = subacute usage level (low level)

SAI = subacute intermediate level (medium level)

SA LD_5 = subacute LD_5 level (MTD level, high level)

CO_2 = carbon dioxide

DMN = Dimethyl nitrosamine

EMS = Ethyl methane sulfonate

TEM = Triethylene melamine

DMSO = Dimethyl sulfoxide

MEM = minimal essential medium (Eagle's)

CPE = cytopathic effect

his = histidine marker

D-3 = mitotic recombinant strain of Saccharomyces

mf = mean mutant frequency

MFt/MFc = mean mutant frequency of the test compound group compared to mean mutant frequency of the negative control group

CFU = colony forming units

WI-38 = code name for a strain of human embryonic lung tissue culture cells

Rec x 10^5 = mitotic recombinants x 10^5

Mean B/A = mean frequency

tot. scr. = total scored

tot. = total

χ^2 = a test of variation in the data from the computed regression line. Tested in these studies at the 5% level

Aberr. = aberrations

Frag. = fragment

HMA = host mediated assay